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Research Article

Chemical Characterization of *Aspergillus flavus* α-Amylase Synthesized in Tobacco Plants Engineered by CRISPR/Cas 9

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ABSTRACT

The endo-type group digestive enzymes, such as α-amylases, play a crucial role in insect carbohydrate metabolism. They primarily catalyze the conversion of starch into structurally simpler carbohydrates by hydrolysis. Despite causing post-harvest damage to grains, such as peanuts and beans, *Apergillus sp*. α-amylases can be used for industrial purposes in food, chemical, and textile modification, adding value to the production chain by increasing the conversion of useful carbohydrates for manufacturing purposes. The amylolytic enzyme studied in this report was biosynthesized in tobacco plants transiently transformed by genome editing using CRISPR/Cas 9 system. It showed specific characteristics when compared to other α-amylases from different organisms, such as unique kinetics, an optimal temperature of 50°C, and an optimal pH (6.0). Starch hydrolysis catalyzed by *Apergillus flavus*. α-amylase was measured using Ionic enhancers and different inhibitors, revealing a decreased amylolytic activity when complexed with metallic ions and organic compounds.

Keywords: α-amylase; Genome edition; CRISPR/Cas9 System; *Apergillus flavus*

INTRODUCTION

Amylases (α-amylase, β-amylase, and glucoamylase) are among the most important products obtained from Biotechnology nowadays. Great amounts of these enzymes can be produced by many microorganisms such as bacteria, yeast, and filamentous fungi, especially from species of the genera *Aspergillus* such as *Aspergillus niger*, *Aspergillus awamori*, and *Aspergillus oryzae* [1,2].

The basic amylolytic property of fungal derived α-amylases (1,4 a-glucan glucanohydrolase, EC 3.2.1.1), is the catalytic conversion of starch into different soluble sugar sub-products, what confers the enzyme an increased interest in food, detergent, chemical, textile, and other industrial purposes [3].

Nevertheless, the starch degradation promoted by the seed crop infesting fungus *Aspergillus flavus* α-amylase (Af α-amylase) confers an important carbon source readily metabolized *via* glycolysis for and is intimately associated with aflatoxins production, having an important role in the induction of these potent liver toxins and carcinogens family of structurally related secondary metabolites biosynthesis [4].

This species has economic importance since it can infect many agronomically important host crops such as cottonseed, carrot, corn kernels, peanut, and tree nuts causing millions of dollars in losses a year worldwide [5-7].

Despite its importance, Af α-amylase is only synthesized in conditions of fungal infection of grains and at low concentrations, which makes it difficult to obtain from natural sources. In this way, recombinant systems of gene expression can become alternatives for the biosynthesis of α-amylases, in sufficient quantities for its use in scientific research and for industrial purposes [8].

In recent years, tobacco plants have proved to be interesting tools for the biosynthesis of recombinant proteins. In addition to being easy to handle, tobacco has the characteristics of a model organism for biotechnology. Its genome has already been sequenced, it has easy genetic transformation and hot spots of gene expression, preferred targets for genome editing, have already been properly mapped [9].

One of the challenges in the use of tobacco as a reactor for recombinant proteins is the randomness in the integration of

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the expression cassette in genomic sites of the plant, which often leads to low levels of transgenic expression [9]. In this context, the CRIPSR/Cas 9 system shows itself as a recommended technology to direct the integration of transgenes to genomic sites associated with high levels of gene expression that allow obtaining satisfactory amounts of protein [10].

The evaluation of the biochemical properties, and kinetic parameters of new related types of Af α-amylase synthesized by genome edited tobacco can provide an increase of efficiency in industrial applications and improve crop storage conditions to avoid degradation of seed storage tissues, loss of agronomical quality, and mycotoxin contamination.

The focus of this report is the evaluation of the biochemical properties of an Af α-amylase, such as optimal temperature and pH for starch degradation, determination of kinetic parameters and ionic and organic compound inhibition, elucidation of protein tertiary structure by a three-dimensional model construction, and analysis of possible amino acid residues directly involved in the catalytic activity of the enzyme.

MATERIALS AND METHODS

Construction of the gene expression vector

DNA amplification by PCR: The amplification of the DNA fragment encoding Af α-amylase (GenBank accession number: AF139925.1), was performed using the primers shown in Table 1 and cloned into the vector p-sgRNA34 (3954 bp), under the control of the cauliflower mosaic virus (CaMV) 35S promoter and octopine synthase (OCS) terminator, upstream of the coding sequence of the guide RNA for genome editing *via* CRISPR/Cas9.

Primer	Sequence	Size	Tm
Alpha-Forward	5'-AGGTATTTACCATGAAGACACGATGTTGAA-3'	30 bp	51°C
Alpha Reverse	5'-AAGCTGAGACCTTAATGATATGGAAGTGT-3'	29 bp	53°C

Table 1: Primer oligonucleotides used to amplify the Af α-amylase coding sequence.

The sgRNA coding sequence, complementary to the last 30 nucleotides of the 5` UTR of the *Nicotiana tabacum* ribulose bisphosphate carboxylase (RuBisCO), the most highly abundant transcript in tobacco [11]. Plasmid p-TruCas9 (6576 bp) was used as the expression vector for Cas9, containing the Cas 9 gene of Streptococcus pyogenes under the control of the folded 35S promoter CaMV and the Tobacco Mosaic Virus (TMV) replicase terminator.

Genetic transformation of *Agrobacterium tumefaciens*: The electroporator (MicroPulserTM Electroporation Appartus, Bio-Rad, USA) was adjusted to the appropriate conditions: voltage of 2.2 kV, capacitance of 25 μF and resistance to 200 Ω (for a cell with 0.1 cm of distance between the electrodes). Then, aliquots containing 45 μL of electrocompetent *Agrobacterium tumefaciens* (GV3101) cell suspension were thawed on ice. 100 ng of DNA related to the recombinant vectors were added to each aliquot. The aliquots containing the mixture of cells and DNA were transferred to the cuvettes, which were inserted into the electroporator and the electric pulse applied. Immediately, the cells were suspended in 1 ml of liquid LB medium; transferred to a 1.5 mL microcentrifuge tube and incubated at 28°C, under agitation of 180 rpm for 90 minutes. Then, 200 μL of the suspension was plated in Petri dishes containing solid LB medium plus the appropriate selection antibiotic (kanamycin (100 μg/mL).

Agroinfiltration of *N. tabacum* leaves: A colony of *A. tumefaciens* from each of the constructions was separately inoculated in 5 mL of liquid LB medium plus kanamycin (100 μg/mL), and then the inoculants were incubated at 28°C with 180 rpm shaking for 48 H. After the incubation period, the cells were collected individually by centrifugation at 7,000 rpm for 4 minutes. Then, the precipitate was re-suspended in 2 mL of saline (infiltration buffer; 10 mM MgCl2) and the OD was measured by means of a spectrophotometer under a 600 nm wavelength (OD 600 nm). Then, dilution calculations were made to produce a cell suspension containing the pro-vectors and recombinant vectors in a final OD of 0.3. Thirty *N. tabacum* plants were used with 15 days of emergence in the soil. Leaves were agroinfiltrated in the abaxial part, using a syringe without a needle.

Extraction of total soluble proteins: Four leaf discs of *N. tabacum* were highlighted for the extraction of total soluble proteins (TSPs), 5 days after agroinfiltration. These were immediately placed in microcentrifuge tubes (2 mL) and stored at -80°C for future analysis. TSPs were extracted using 4x (m/v) of the extraction buffer (50 μM Tris HCl, pH 7.5; 150 μM NaCl; 0.1% Tween-20; 0.1% β-mercaptoethanol) [12-14], plus 10% (v) of proteinase inhibitors (PIC; Sigma-Aldrich, Missouri, United States). The leaf discs were macerated with the aid of metal spheres, under agitation of 30 Hertz in a tissue processor (Tissuelyser II, Quiagen, Hilden, Germany) for 3 minutes. Then, the samples were centrifuged at 8,000x g for 15 minutes at 4°C and the supernatant was transferred to a new microcentrifuge tube (1.5 mL). The samples were clarified by means of a new centrifugation at 8,000x g, for 10 minutes at 4°C, and the supernatant transferred to a new tube.

α-amylase purification: Protein extracts (20 mg/ml) were applied into an affinity chromatography Sepharose-6B conjugated with β-cyclodextrin equilibrated with 0.1 M phosphate buffer, pH 7.2, containing 20 mM NaCl and 0.1 mM CaCl2. Positively charged proteins were collected from previously numbered tubes 1 to 17. After the addition of 20 ml of 1 M of NaCl into the column, proteins with negative charge were eluted and collected in tubes 18 to 44. Fractions (2.0 ml) were collected at a flow rate of 28 ml h^{-1} and used to measure a-

amylolytic activity. Retained fractions were pooled, concentrated with TCA (Trichloroacetic Acid) 12%. After having purified and selected the proteins for changes, it went measured to absorbance (280 nm) in the spectrophotometer, detected of the purified enzyme and was analyzed by SDS-PAGE mini-gels 15% at a standard concentration of 10 mg.ml^{-1} according to Laemmli (1970).

Enzymatic assays: We used Bernfeld protocol (1955), to measure Af α-amylase. Enzyme activity was evaluated under concentrations of 2 mg/ml, diluted in Tris-HCl buffer 0.05 M, pH 7.0. Starch (1% w/v) was added to the reaction as substrate. Each fraction was incubated at 37°C for 20 min. The enzymatic reaction was stopped by adding 1.0 ml of 3.5 DNS (1% dinitrosalicylic acid dissolved in 0.2 M NaOH and 30% sodium potassium tartrate) and was evaluated by optical density at 530 nm. Each assay was carried out in triplicate.

Biochemical characterization of *Aspergillus flavus* α-amylase: The determination of Af α-amylase biochemical properties was carried out under a standard concentration of 20 mg.ml^{-1} diluted in 0.05 M Tris-HCl buffer, pH 7.0.

The structural stability and activity of the enzyme were evaluated by catalytic assays under different pHs and temperatures. Triplicate samples conatining α-amylase (2 mg/ml-20 µl) were submitted to treatments with 0.05 M Tris-HCl buffer under acidic pHs (pHs 4,5 and 6), neutral (pH 7) and basics (pHs 8, 9 and 10), and different temperatures (25°C, 30°C, 40°C, 50°C, 70°C and 90°C/10 min). After incubation, 100 µl of starch 1% (w/v) was added to each sample, and incubated for 20 min at 37°C.

The modulation of the enzymatic activity was measured using 14 ions and organic compounds, such as: Acetone, AgCl2, AgNO3, CaCl2, CuSO4, FeCl3, Glicine, KCl, KNO3, MgCl2, MnCl2, NaCl, Proline and ZnSo4 with final concentrations of 0.05 mg.ml^{-1}. Samples were composed by enzyme (2 mg.ml^{-1}), the respective ion solution (0.05 mg.ml^{-1}), Tris-HCl buffer (50 mM), pH 7 and starch 1% (w/v), and incubated for 20 min at 37°C. All samples were carried out in triplicate.

Kinetic parameters of *Aspergillus sp.* α-amylase: Starch enzymatic hydrolyzation was carried out according to Tester. For evaluation of the kinetic parameters of Af α-amylase. Samples (20 µl) from reaction mixture containing Af α-amylase and also with substrate concentrations (0,33%; 0.30%; 0,26%; 0,23%; 0,20%;0,16%; 0,13%; 0,10%; 0,06%; 0,03% and 0,01%) were dissolved in 50 mM Tris-HCL buffer (pH 7.0) at 37°C/20 min. The reactions were immediately stopped by adding dinitrosalicylic acid (0.1% v/v) and incubation for 20 min at 95°C. The amount of reducing sugars produced from soluble starch was measured according to Lindsay (1973). Kinetic data were transformed to Lineweaver-Burk plots and Km values were calculated from the slopes of the curves.

RESULTS

Biochemical characterization of *Aspergillus sp.* α-amylase

For the evaluation of Af α-amylase biochemical properties, enzymatic samples were submitted for purification and loaded onto an Epoxi-Sepharose 6B affinity column. A chromatographic profile was obtained from protein elution by a single step of NaCl 1 M, (Figure 1).

Fractions with larger peaks observed in the graphic (tubes 8,9,21,22,23,24,25,26 and 39) were selected and stored at 5°C. Tube 39 showed the highest level of absorbance at 530 nm and its contents were resolved by SDS-PAGE mini-gel 15%, at concentrations of 2, 4, 6, 8, and 10 µg.ml^{-1} as shown in Figure 2.

Purified α-amylase sample presented molecular mass near 52 kDa as observed using SIGMA's low mass protein marker and related previously in the literature.

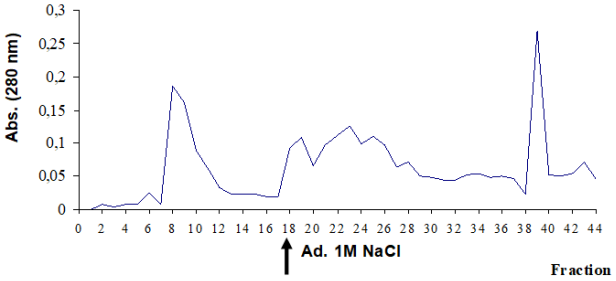

Figure 1: Chromatographic profile obtained during the purification of Af α-amylase using EpoxiSepharose 6B chromatography collumn equilibrated with 0.1 M phosphate buffer, pH 7,2, containing 20 mM NaCl and 0.1 mM CaCl2. The black arrow indicates the single-step application of 1 M NaCl.

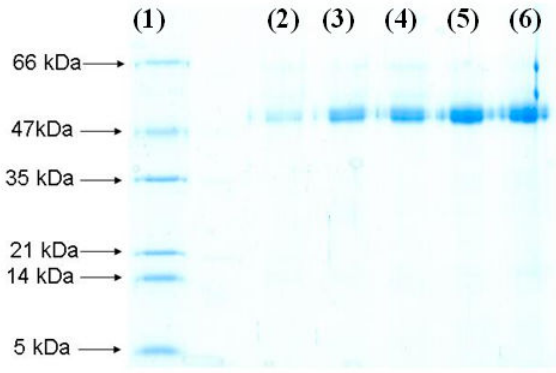

Figure 2: Electrophoretic profile of Af α-amylase showing an approximated mass of 52 kDa resolved purified fraction. 1 shows 7 µl of SIGMA's low mass marker. 2, 3, 4, 5, and 6 show respectively 2, 4, 6, 8, and 10 µg.ml of the purified fraction by affinity chromatography.

Kinetics parameters of amylolytic activity were determined by a reaction ratio vs. substrate concentration curve, which produced a Michaelis-Menten kinetic profile, indicating a hyperbolic curve represented by an increase in starch hydrolysis until a substrate saturation point remarked by enzymatic activity stabilization (Figure 3).

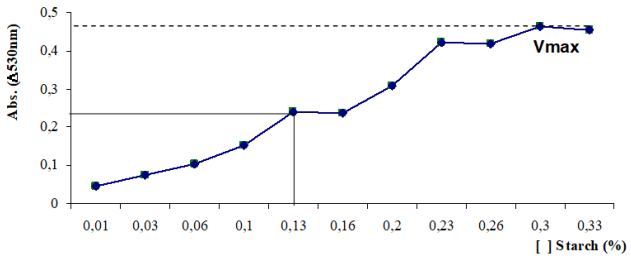

Figure 3: Enzymatic activity by a variation in starch concentration. An accentuated increase in amylolytic activity is observed until the starch concentration reaches 0,23%, followed by the curve`s stabilization. The calculated Km ([S]=1/2 Vmax) for starch was 0,13% (w/v).

The construction of a thermostability curve showed higher α-amylolytic activity under 25 to 50°C (Figure 4A). The enzyme structural stability was maintained until 45°C as suggested by Figure 4B, getting a pronounced decrease after 55°C. Around 70°C it is possible to observe both decreases of activity and structural stability, showing that under this temperature denaturation process is taking an advanced course.

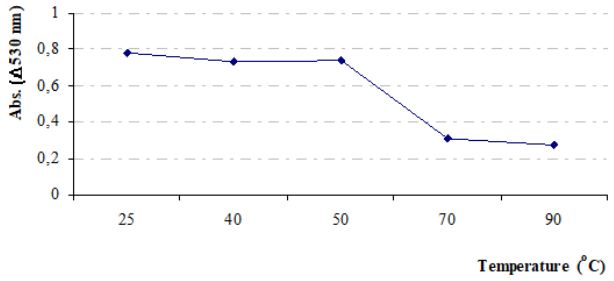

Figure 4: (A) Effects of temperature on Af α-amylase amylolytic activity.

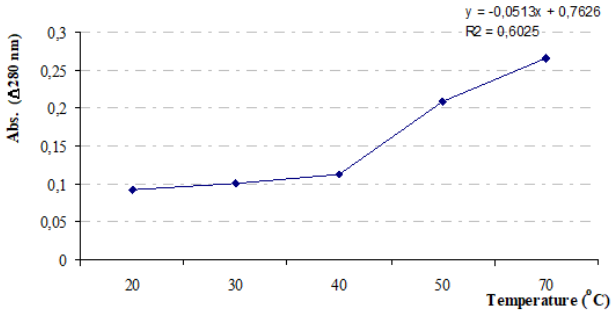

Figure 4: (B) Structure stability.

The denaturation process results in aromatic hydrophobic residues exposition, increasing the absorbance ratio greatly after 50°C, corroborating with the decrease of amylolytic activities. The highest Af α-amylase activity was observed under pH 6.0 (Figure 5A), decreasing greatly after pH 7.0. Lower activities occurred under acidic pH 4.0 and under alkaline pHs (9.0 and 10.0).

The decrease of enzymatic activity at pH 7.0 corroborates with the greater absorbance ratios under this condition, indicating a less-stable protein form under neutral pH like suggests in Figure 5B.

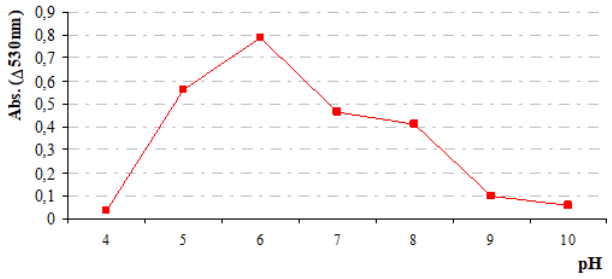

Figure 5: (A) Af α-amylase amylolytic activity.

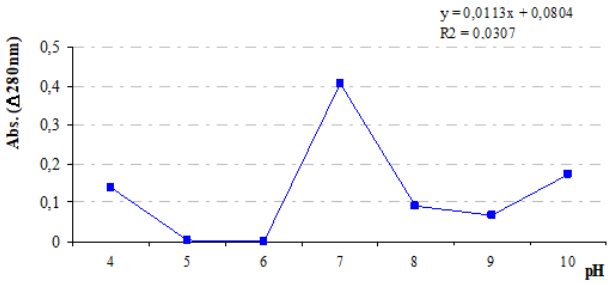

Figure 5: (B) Structural stability under different pH conditions.

Af α-amylase amylolytic activity was tested in the presence of ions and organic compounds Acetone, AgCl2, AgNO3, CaCl2, CuSO4, FeCl3, Glicine, KCl, KNO3, MgCl2, MnCl2, NaCl, Proline, ZnSO4. All substances decreased amylolytic activity, specially AgNO3 and Glicine. Acetone was responsible for the sweetest decrease, followed by CaCl2, NaCl, MnCl2, FeCl3, KNO3 and AgCl2 (Figure 6).

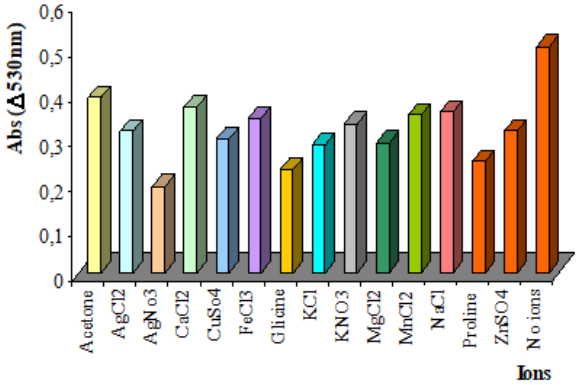

Figure 6: Ionic and organic compounds effects on Af α-amylase amylolytic activity.

DISCUSSION

This report determined several properties for Af α-amylase. According to our results, the enzyme showed higher activity between 25 to 50°C, and the lowest activity at 90°C, which suggests the effect of thermal denaturation and loss of specificity and catalytic efficiency by the enzyme.

Denaturation may cause the exposure of hydrophobic amino acids that were hidden inside the internal surface of the active site and were crucial for catalysis and protein stabilization as tryptophan and phenylalanine [15].

Thermal stability (between 20 and 40°C) and optimum temperature for catalytic activity (near 40°C) for this Af α-amylase reflects similarities between the enzyme and α-amylase isolated from bean plague *Acanthoscelides obtectus* [16].

Also, similar results for the activity of an α-amylase from Peruvian carrot (*Arracaia xanthorrhiza* Bancroft.) under optimum pH of 6.0 were found by Pires et al., 2002. Both enzymes, from *Aspergillus sp*. and Peruvian carrot has a pH range between 5.0 and 7.0, that still confers catalytic activity [17].

What makes this Af α-amylase interesting for industrial purposes is the maintenance of the catalytic efficiency under a wide range of pH without abrupt decrease, which is desirable for long-term carbohydrate hydrolysys [18].

The pH range observed for the enzymatic activity of *Rhizopus oligosporus* α-amylase varied from 3.0 to 5.5, significantly different from our observation in *Aspergillus sp* [19]. This indicates variation between the two fungi enzymes on the ionized ratio of catalytic functional groups from residues presented in the internal surface of the catalytic site.

In the presence of al ions and organic compounds tested Af α-amylase showed a lower activity ratio than in absence of these substances. Differently from what Dutta et al., 2005 have observed, since crustacean *Heliodiaptomus viduus* (Gurney) [20]. the α-amylase amylolytic activity showed a great enhance in the presence of metal ions like $Fe2^{+}$, $Ba2^{+}$, $CO2^{+}$, $Ag2^{+}$, and $Mn2^{+}$, up to 130%-200% of the original activity. Otherwise, in the presence of $Cu2^{+}$, $Mg2^{+}$, Amylase activity was completely inhibited like in Aspergillus sp., but in a more drastic way than ours, preserving only 5% of the original activity.

CONCLUSION

The structural variability between α-amylases found in nature can explain the variation in the activity profiles between these enzymes in a great range of organisms. This is particularly interesting when α-amylases from individuals from different species under the same kingdom show less kinetic similarity than that observed organisms of different kingdoms.

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